METAMORPHOSIS OF THE VELIGER LARVAE OF NASSARIUS OBSOLETUS (GASTROPODA) IN RESPONSE TO BOTTOM SEDIMENT [1]

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Characteristic of most marine organisms is propagation of their kind by the production of great numbers of pelagic larvae. As a consequence, the dispersion of such organisms over large areas is possible. Success of benthic organisms is dependent, however, upon the metamorphosis of their larvae onto a substratum which meets their special adaptations and requirements. The metamorphosis of marine larvae upon a favorable substratum, if left to chance alone, would be indeed remote.

This hazard is apparently not as great as was once supposed, since many marine invertebrate organisms delay metamorphosis until they encounter a favorable substratum. Metamorphosis occurs as the response to a stimulus received from the desirable environment. Inasmuch as the evidence has been reviewed in a recent paper by Wilson (1952), it is necessary to relate only briefly the facts which lead to this conclusion.

The earliest laboratory observations which indicated that the substratum played a role in stimulating metamorphosis were those of Mortensen (1921) on the echinoid *Mellita sexiesperforata*. Mortensen made the casual observation that when larvae which were ready to metamorphose were placed in a culture jar with sand, metamorphosis ensued within a few days, while similar larvae from the same original culture placed in a jar without sand showed "hardly any further advance in the process of metamorphosis." This observation was later confirmed on three other species (Mortensen, 1938).

Harrington (1921) observed that shipworm larvae (*Teredo norvegica*) were positively chemotactic to a substance which he extracted from wood and which he believed was malic acid. Wilson (1952) has extended this observation and has shown that in the absence of the wood, shipworm larvae do not metamorphose. Settlement may be postponed for about one week.

Wilson (1932, 1937, 1948) and Day and Wilson (1934) observed the response of larvae of several polychaetous worms to the presence of their natural substratum. In all cases the settlement of the larvae was brought on by the addition of small quantities of substratum collected from their normal habitat. Substrata from other areas either were less effective or appeared to inhibit metamorphosis. Wilson chose *Ophelia bicornis* for a more detailed study and the results of this work have been reported in a series of papers (Wilson, 1948, 1951, 1952, 1953a, 1953b, 1954,

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1955). While size and shape of grains seemed to have some bearing on the metamorphosis-inducing quality of the substratum, Wilson (1955) finally concluded that (p. 537) "the factor most active in inducing metamorphosis and settlement of *Ophelia bicornis* larvae is the presence on sand grains of living organisms, such as bacteria, and that these should be neither too few nor too abundant. . . . Certain species are more effective in promoting settlement than are others. . . . "

Two other species of polychaetes have shown a settlement response to the presence of substratum. Nyholm (1950) found that *Melinna cristata* (p. 88) "tended to metamorphose only in the presence of a suitable substratum rich in detritus and never when merely supplied with clear water." Smidt (1951) observed that (p. 60) "the lack of substratum will prolong larval life [of *Pygospio elegans*] while settling is furthered by the presence of a substratum." Moreover, "sterile sand and naturally occurring pebbles have an impeding effect" on metamorphosis.

In a careful study, settling of the actinotroch larvae of *Actinotrocha branchiata* [= *Phoronis mülleri*] is described by Silén (1954, p. 234): "The metamorphosis is easily and infallibly brought about by introducing some of the clay-mixed sand in which the adults are found, to a bowl containing a mature specimen." The metamorphosis which occurs "within a few usually about ten minutes" is described in great detail and involves the testing of the substratum by the larvae.

The study of Jägersten (1940) on the metamorphosis of the archiannelid *Protodrilus rubropharyngeus* is especially interesting because it describes the characteristics of a metamorphosis-inducing material. In nature *P. rubropharyngeus* has positively phototactic mature swimming larvae which, on reversing their photic response, sink to the bottom and metamorphose in a limited littoral zone with a shell or shell and pebble bottom. In the laboratory swimming larvae usually do not metamorphose but may live for two weeks without signs of transformation. If a shell or pebble from the normal habitat is included in the culture vessel, the larvae metamorphose within twenty-four hours. The material which induces this metamorphosis may be transferred to the water. It is resistant to heat, acids, and alkalis and is apparently associated with suspended material which may be removed either by centrifugation or filtration. Jägersten concluded that some non-living inorganic material is responsible for the settling response.

Wilson (1952) has concluded (p. 53 ff.): "It is evident that larvae do not settle just anywhere and that many of them have some power of selection. Over what period of time this power can be exercised is not always so clear; neither is the mechanism whereby larvae are enabled to make the selection, whether by touch, smell or other means. . . . It has proved easier to establish the facts that metamorphosis can be delayed and that larvae can choose their substratum . . . than to determine the actual features of the environment, physical, chemical or biological, perceived by the larvae and to which they react."

One of the more conspicuous organisms on intertidal flats along the Atlantic coast from the Gulf of St. Lawrence to northern Florida is *Nassarius obsoletus* Say. This prosobranch gastropod is extremely abundant in its preferred habitat. Newly metamorphosed snails, during favorable years, occur in large aggregations which reach densities of 23,000 individuals per square meter. A patch of newly metamorphosed snails, while usually not uniform in density, may exceed 100 square meters in area. Apparently success of the species is due to the settlement of the larvae in a favorable environment; an explanation may be that the larvae metamorphose in response to some clue provided by the environment.

The experiments which are described in the following pages are offered in support of such a hypothesis. They show that in the laboratory, the larvae of the gastropod mollusk *N. obsoletus* are able to recognize a favorable substratum and can, to a considerable degree, delay metamorphosis until a suitable substratum is come upon. Hence the "power of selection" in choosing a place for settlement is proven to exist in yet another group of invertebrate animals. The experiments also suggest the singular manner by which the larvae achieve this end.

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Experimental Procedures

The experiments that have demonstrated the relationship between the substratum and metamorphosis in *Nassarius obsoletus* have been simple in their design. Inasmuch as all of these experiments involved quite similar methods, the general procedure used will be described here; the details are presented later together with the results.

All experiments entailed the use of at least one control and one experimental container, each with twenty veliger larvae. The containers, which were of translucent polyethylene,[3] were filled with one-half liter of sea water that had been filtered through glass or dacron wool.

The bottom sediment to be tested was placed in the experimental dishes. About 5 cc. of substrate material were required to just cover the bottom of a container. The substratum, unless otherwise indicated, was screened before use by passing it through either a Number 60 (240 μ) or 100 (140 μ) stainless steel screen, or a Number 2 (316 μ) bolting cloth. This removed all organisms larger than *ca.* 0.3 mm, which interfered with the conduct of the experiment. The substratum usually was composed of a very fine sand and included a considerable amount of organic material.

The controls consisted of containers with filtered sea water only; in some cases, when the substratum was treated in a manner to be explained later, additional controls with untreated substratum were added to the series.

[3] Manufactured by the Kordite Corporation, Macedon, N. Y. Polyethylene to which coloring matter has been added is unsuitable, presumably because of the toxic effect of the dye.

Control and experimental containers were started simultaneously with larvae grown in and picked at random from the same culture. The larvae at the beginning of the experiment were at a stage of development which precedes metamorphosis, *viz.*, the creeping-swimming stage, attained about 3 weeks after emergence from the egg capsule. Subsequently both experimental and control containers were placed together on a sea table or nearby shelf. Hence the larvae are presumed to have been maintained under similar but not necessarily constant or uniform conditions throughout the course of their growth previous to the experiments and also, except for the conditions being tested, throughout the duration of the experiments. Temperature throughout the experiments ranged from 22° to 24° C.

Most experiments were terminated after 24 hours; some ran only 12 hours and others lasted up to four days.The larvae or recently metamorphosed young were removed from the plastic containers either by pipetting them directly from the containers or by carefully screening the contents of the containers so as to permit the substratum to pass through, leaving the organisms behind. The number of larvae that had metamorphosed was then determined by inspecting each larva under a dissecting miscroscope. The criterion used to determine metamorphosis was the loss of the velum.

A concise description of the method used in rearing the larvae will suffice, inasmuch as a more complete account will be given elsewhere, together with the external morphology of the larvae. Egg capsules were obtained in the field or from snails held in the laboratory. After emergence from the egg capsules the larvae were collected on a fine mesh screen, placed into 15-liter rearing vessels, and aerated. Water in the rearing vessels was changed every second or third day by passing the entire contents of the vessel through a fine mesh screen which retained the larvae. *Phaeodactylum tricornutum* Bohlin (Ehr.) W. Sm. at a concentration of 2×10^5 cells/ml. was used as a source of food. This diatom may be readily grown in mass culture.

A brief explanation of the experimental data given in the tables is required. Since the age of the larvae, as well as previous cultural conditions, was of necessity not identical from one group of experiments to the next, any set of experiments performed at one time are not strictly identical to those of another. The past history of the culture and the difference in the age of the creeping-swimming larvae explain much of the variability observed in the data. It is clearly valid, however, to compare the results from a series of experimental containers with those derived from their respective controls. The statistical significance of the difference between such means has been determined by the Student t-test. The percentages metamorphosed were corrected for mortality, and all statistical computations are based on the number of living organisms recovered at the end of the experiments.

Reaction of Nassarius obsoletus to the Substratum

a. *Metamorphosis in the presence of natural substratum*

The importance of the substratum to onset of metamorphosis in *Nassarius obsoletus* is readily demonstrated. In the presence of a bottom sediment on which

TABLE I

Percentage metamorphosis of Nassarius obsoletus *in the presence of of natural substratum (results from ninety-four experiments)*

Experimental condition	Per cent mortality	Mean per cent metamorphosed*
Natural substratum	2.1	70.0 ± 2.4
Without substratum	1.4	18.9 ± 1.4

* One standard error is indicated on all values throughout this paper.

juvenile *N. obsoletus* are found in nature, the percentage metamorphosis is very significantly greater than in controls without such natural substratum. The results of 94 experiments show that with a natural substratum 70.0% of the larvae ended their planktonic existence, whereas in controls of filtered sea water only, 18.9% of the larvae metamorphosed. The value of P in these experiments equals less than 0.001. Moreover, in not one of the above experiments did the percentage of metamorphosis in the control ever exceed that in the experimental culture. The results of the experiments are summarized in Table I. The larvae at the end of

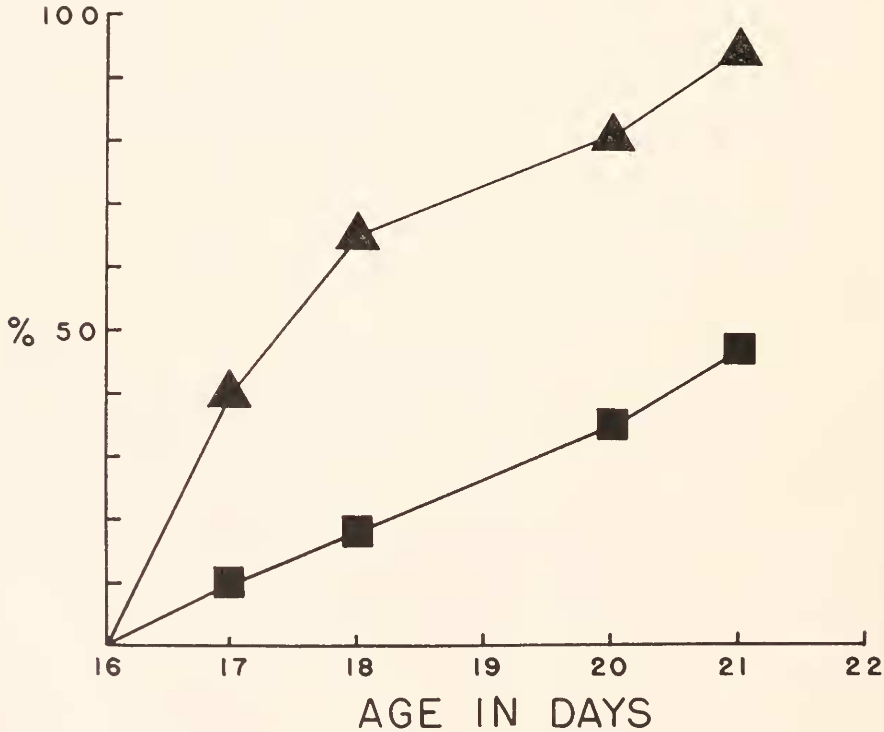

FIGURE 1. Percentage metamorphosis with (▲) and without (■) natural substratum in *Nassarius obsoletus*. Fifty larvae, 16 days after emergence from egg capsules, were placed in each of two 2-liter containers. In the bottom of one was placed freshly collected substratum; no substratum was placed in the control. The percentage metamorphosis in each container is shown at the times indicated on the abscissa.

the experiments ranged in age from 16 to 35 days (mean 25.7) after emergence from the capsule. The eggs were obtained from snail populations in the vicinities of Woods Hole, Massachusetts, and Beaufort, North Carolina.

Apparently metamorphosis cannot be indefinitely delayed. In the absence of bottom sediment the tendency to metamorphose is a simple linear function of age (Fig. 1). The maximum delay which was demonstrated was 20 days after the creeping-swimming stage had been attained. In the presence of bottom sediment the tendency to metamorphose is also a function of age; however, this tendency increases very rapidly and is not a simple linear function. In a series of experiments (Scheltema, 1956), it was shown that when 20-day-old larvae were used, 17% metamorphosed in response to bottom sediment, whereas in similar experiments using 31-day-old larvae 90% metamorphosis resulted.

TABLE II

Mechanical analyses of substrata from three areas used in metamorphosis experiments*

Mesh no.	Openings in mm.	Per cent passing through sieve		
		Substratum #1	Substratum #2	Substratum #3
5	4.000	82.8	—	—
10	2.000	75.4	—	—
18	1.000	57.5	100.0	100.0
35	0.500	24.3	99.6	97.7
60	0.250	6.3	94.4	92.0
120	0.125	0.5	66.2	84.1
230	0.062	—	7.3	77.3
Median diameter		0.9 mm.	0.1 mm.	<0.06 mm.

* Substratum #1: Head of Great Pond (off mouth of Perch Pond), East Falmouth, Cape Cod, Massachusetts (see Hulburt, 1956). "Favorable" substratum in preference experiments.

Substratum #2: Experimental area of Woods Hole Oceanographic Institution, Barnstable Harbor, Cape Cod, Massachusetts (see Ayers, 1959).

Substratum #3: Mouth of Great Pond (west shore), East Falmouth, Cape Cod, Massachusetts (see Hulburt, 1956). "Unfavorable" substratum in preference experiments.

b. *Preference for a favorable substratum*

Certain natural substrata are apparently preferred over others, inasmuch as a significant difference in the response of *Nassarius obsoletus* larvae to different substrata may be demonstrated experimentally. A sustratum sample obtained from a location shown to be very favorable for metamorphosis of *N. obsoletus* (#1, Table II), as indicated by the large number of snails observed to have metamorphosed there in the previous two years, was compared with another sample collected from a region believed to be unfavorable for metamorphosis (#3, Table II), as demonstrated by the complete absence of recently metamorphosed snails during the same two-year period. The latter region, although unfavorable for metamorphosis, was not inimical to the existence of the species, since older snails of three years or over regularly were found to inhabit this flat. Plankton tows revealed great numbers of larvae during the months of July in the waters over both regions from which the substratum samples were obtained.

The two substrata differed markedly. That taken from the area where juvenile snails were abundant was composed largely of coarse sand and fine gravel. It was poorly sorted, with a median grain size of 0.9 mm. An abundance of living diatoms was present in this substratum. The other substratum was rich black and probably somewhat anaerobic since a trace of hydrogen sulfide odor could be detected. This material was less than 0.06 mm. in median diameter and may be characterized as silt. Viewed under the microscope it appeared to contain large amounts of organic detritus. The mechanical analyses of the substrata are summarized in Table II. The substrata used in the experiments were not screened, but all larger organisms were removed from them with forceps under a dissecting microscope.

Table III

The preference of Nassarius obsoletus *for a favorable substratum for metamorphosis (results from seven experiments)*

Experimental condition	Per cent mortality	Mean per cent metamorphosed
Favorable substratum (#1, Table II)	0.0	84.4 ± 8.3
Unfavorable substratum (#3, Table II)	0.0	51.6 ± 7.6
Without substratum	0.0	30.2 ± 11.0

The percentage metamorphosis on the two substrata may be compared in Table III. In the coarse substratum 84.4% of the larvae metamorphosed, whereas on the fine black organic substratum 51.6% ended their planktonic existence. This difference is statistically significant (P equals less than 0.025). The difference between the fine black substratum and the control without substratum was not significant (P equals 0.20). The mean age of the larvae at the beginning of the experiment was 25.0 days.

The experiments show that substrata favored in the laboratory are similar to those preferred under natural conditions, and that there is a correspondence between experimental results and field observations. The general application of these results should be viewed with considerable reservation, however, pending further experiments using other favorable and unfavorable bottom sediments.

Properties of the Substratum Which Induce Metamorphosis

Numerous experiments indicate that the induction of metamorphosis by a favorable substratum is not a direct function of its physical character, but rather the result of its biological and chemical properties. Two lines of evidence support this conclusion.

a. *Physical properties of the substratum*

During the course of many experiments, sediments have been used from widely diverse regions. Mean grain size and sorting have differed greatly. Of those substrata that have been tested experimentally and proven effective in inducing metamorphosis, the greatest extremes in physical properties are to be found in substrata #1 and #2 in Table II. The former, from the head of Great Pond, East Falmouth, Massachusetts, is poorly sorted gravel and sand. The latter, from Barnstable Harbor, Cape Cod, Massachusetts, is well sorted and consists primarily of fine reworked glacial sand. For the purpose of the experiments, the Barnstable Harbor substratum was passed through a Number 2 (316 μ) bolting cloth. This sieving had little effect on the physical character of the sediment, removing at most perhaps 2% of the total sample, *i.e.*, that portion exceeding 0.3 mm., in diameter. The Great Pond sample was not screened, but mollusks and annelids were removed. Table IV shows the results obtained with these substrata: in the Great Pond sample 84.4% of the larvae metamorphosed, while in the Barnstable substrate 88.7% completed their planktonic existence. Although experiments with the Barnstable and Great Pond sediments were conducted at different times and with larvae from different cultures, it seems justifiable in this instance to compare results and to conclude that the wide difference in physical character of the substrata had no

TABLE IV

The effect of two substrata, differing widely in physical properties, on the percentage metamorphosis of Nassarius obsoletus

Substratum used	Character of substratum	No. of expts.	Mean per cent metamorphosed with substratum	Mean per cent metamorphosed without substratum
Great Pond, Falmouth, Mass. #1, Table II	0.9 mm. median diameter, poorly sorted sand and gravel	7	84.4 ± 8.3	30.2 ± 11.0
Barnstable Harbor, Mass. #2, Table II	0.1 mm. median diameter, well sorted fine sand	18	88.7 ± 3.3	21.0 ± 4.8

effect on their ability to influence metamorphosis. The experimental results are completely in accord with the observations in nature that the larvae in fact do metamorphose in large numbers on these two substrata.

It may be concluded from this evidence that, within wide limits, mean particle size and sorting have no direct effect on metamorphosis. There are, however, certain indirect effects of particle size on the biological properties of the substratum as a consequence of decreased surface with increasing particle size. ZoBell (1946) has shown, for example, that the size of bacteria populations may be related to particle size.

b. *Biological and chemical properties of the substratum*

Response of larvae to the biological and chemical attributes of the substratum was tested in a series of experiments. It was intended to alter the sediment biologically and chemically without changing its physical properties.

Treating a substratum sample by (1) incinerating, (2) oven-heating, (3) washing, (4) heating in a water bath, or (5) irradiating with ultraviolet light results in each case in a significant reduction of the metamorphosis-inducing properties of the substratum.

Incineration was accomplished by first washing and boiling the sediment in distilled water several times to remove most of the organic material, and then placing the cleaned sediment in a crucible and heating it above the high flame of a bunsen burner for one hour. The result of this treatment was to render the substratum abiotic and to free it of all traces of organic matter.

Oven-heating the sediment was done at 210° C. for 15 to 30 hours. The dried sample when removed from the oven had a dark brown appearance and a slightly pungent odor. This substratum also was abiotic, but differed from the incinerated substratum in probably having some organic material still present.

Washing the sediment consisted of boiling it in sea water and then in distilled water for several minutes, stirring vigorously, and finally decanting the surplus fine organic material. This process was repeated until the sample appeared essentially clean, *i.e.,* until it assumed a more or less light gray color and lacked any apparent suspended material when stirred. This treatment resulted in removal of part of the organic material and perhaps much of the natural microflora.

Heating the sediment was done by placing a small flask containing some sea water and the sediment sample into a beaker of water and raising the temperature to 70° to 90° C. for five to fifteen minutes. When the experiments were performed, the sea water heated inside the flask with the substratum was used along with new sea water added to make up a volume of one-half liter. Hence no part of the original substratum could have been lost, even if some material is assumed to have gone into solution as a result of the treatment. It was hoped that as a result of this treatment most of the micro-organisms would be killed without substantially affecting the organic material in the sediment. Actually this manipulation resulted, after a period of 24 hours, in a greater total number of micro-organisms than found in the natural substratum, presumably owing to the increased growth of saprophytic bacteria on dead micro-benthos. The difference amounted to two orders of magnitude as determined by plate counts, using ZoBell's marine medium No. 2216 (ZoBell, 1946). The resulting micro-flora was also qualitatively different. The treatment did not appear to cause anaerobic conditions and no fouling of water occurred.

Irradiation with ultra-violet light was done by placing, under a 15-watt germicidal lamp, containers with sediment covered by sea water to a depth of $1\frac{1}{2}$ cm. The treatment was continued 18 hours, the containers being occasionally agitated. After treatment additional sea water was added to each container. Preliminary studies showed that UV treatment reduced the number of bacteria culturable in a marine medium (ZoBell's 2216) by two orders of magnitude if plated after two hours of treatment. The results of the UV treatment were not entirely successful in that complete sterilization is not possible. There is a minimum bacterial count below which further treatment is ineffective even with frequent agitation. Moreover, subsequent to irradiation, growth of saprophytic bacteria is probably very great because of the increase in decomposable material (dead micro-benthos).

The five treatments, as is shown in Table V, all resulted in a significant reduction in the attractiveness of the substrata, as determined by their effectiveness in promoting metamorphosis. Comparison of the experimental groups with their

TABLE V

The effect of various treatments on the metamorphosis-inducing property of the substratum

Treatment of substratum	No. of expts.	Mean age of larvae at start	Mean per cent metamorphosis in treated substratum	Mean per cent metamorphosis in control (untreated substratum)	Difference between treated substratum and control
Incineration	14	24.6	7.2 ± 1.5	71.2 ± 5.3	64.0
Oven-heating (210° C.)	11	26.8	19 0 ± 4.5	69.7 ± 5.0	50.7
Washing	10	22.8	4.6 ± 1.2	51.5 ± 3.9	46.9
Heating in water bath (70°–90° C.)	24	28.4	40.0 ± 4.7	81.2 ± 3.8	41.2
UV irradiation (18 hrs.)	5	28.0	70.4 ± 6.7	91.4 ± 3.4	21.0

untreated controls gives values of P less than 0.001 in all cases except that of the UV irradiation, in which a value of 0.05 was obtained.

Inasmuch as all the treatments shown in Table V have in common the fact that they drastically affected the biological properties and to varying degrees the chemical properties of the substrata, without greatly affecting the physical properties, one may conclude that biological and chemical attributes of the substrata are responsible for their metamorphosis-inducing property.

The various treatments of the substrata in these experiments (Table V) cannot be directly compared with one another because the experiments were conducted with different samples of sediment and with larvae of different ages from different cultures. Nevertheless, the differences between the treated and the control substrata (right-hand column) give some suggestion as to the relative effectiveness of each treatment in destroying the metamorphosis-inducing property of the substrata.

Further experiments have confirmed that a statistically significant difference does indeed exist between the various treatments of the substrata and their attractiveness in inducing metamorphosis. The experiments shown in Table VI were performed with larvae from the same culture and with substratum from a single source. Their only differences were the experimental conditions indicated in the left-hand column. Heating of the substratum in a water bath to 90° C. for

TABLE VI

Relative effect of two treatments of the substratum on its metamorphosis-inducing property (results from fourteen experiments)

Experimental condition	Per cent mortality	Mean per cent metamorphosed
Untreated substratum	1.5	71.2 ± 5.3
Substratum heated in water bath to 90° C.	3.4	33.3 ± 4.8
Incinerated substratum	0.0	7.2 ± 1.5
Without substratum	1.6	13.9 ± 2.6

15 minutes rendered it very significantly less attractive than the normal substratum; however, it also remained very significantly more attractive than incinerated substratum or the controls without substratum (P equals less than 0.001 in both instances). No significant difference resulted between incinerated substratum and the controls without substratum (P equals approximately 0.10).

These experiments show that the treatment of heating the substratum in a water bath at 90° C. yielded results which fall approximately midway between those using the natural substratum and the incinerated substratum.

The experiments on the effects of various treatments of the substratum indicated that: (1) complete removal of the micro-flora and -fauna and of all organic material rendered the substratum unsuitable for stimulating metamorphosis of veliger larvae; (2) qualitative and quantitative changes in the bacterial flora, accompanied by (a) the removal of living microscopic algae and micro-fauna and (b) the alterations in the organic material, owing to heat and addition of decomposition products from the micro-benthos, made the substratum less attractive in inducing metamorphosis; (3) the biological and possibly the chemical character of the substratum was the determining factor in making the substratum suitable for metamorphosis.

A micro-benthic community cannot be separated from the organic material in a sediment. Therefore there is no experimental procedure by which to measure the effect of a micro-benthic community as a whole upon the metamorphosis-inducing property of the substratum. Single species isolated from the substratum may be tested if members of the micro-benthos can be isolated. Preliminary experiments on the effects of benthic marine bacteria isolated from favorable substrata have not yielded consistent results. The effect of the organic material on the suitability of the substratum theoretically may be tested, but no satisfactory method is available to sterilize a substratum without altering the organic material present.

Nature of the Metamorphosis Response: Experimental Evidence for the Presence of a Soluble, Biologically Active, Substratum Factor

How do the larvae perceive a favorable substratum? Is actual contact with the substratum necessary or can the larvae distinguish a favorable place for metamorphosis by some sensory clue received from material liberated into the water? Is some biologically active substance or perhaps ectocrine (see Lucas, 1955) responsible for the response to the substratum? The experiments which follow were designed to answer these questions.

a. *Transfer of the metamorphosis-inducing factor from the substratum to adjacent water*

When sea water is permitted to stand over a substratum favorable for inducing metamorphosis of veliger larvae, it soon acquires some of the metamorphosis-inducing property. Evidently some of the qualities of the substratum are transferred to the sea water.

The water was conditioned by filling a large fingerbowl to 2 cm. in depth with a sediment favorable for metamorphosis. The remainder of the bowl was then filled with fresh, filtered sea water and allowed to stand in a south window for several days to a week. Carefully the sea water was then siphoned off, particular care being taken to exclude particles of the sediment. Filtered sea water not treated with substratum was used as control. A total of ten different substratum samples was used. In some cases, because of the time involved in treatment, the conditioned sea water was re-used. The results of the experiments are shown in Table VII.

The mean percentage metamorphosis in the substrate-conditioned sea water was 48.5 and differed significantly from the untreated control in which only 11.2% of the larvae metamorphosed (*P* equals less than 0.001).

The results showed that a metamorphosis-inducing factor could be transferred from the substratum into the water to a significant degree, and that larvae were able to discriminate between water recently exposed to a favorable substratum and water not recently in contact with such a substratum.

TABLE VII

The effect of sea water conditioned with substratum on the percentage metamorphosis of Nassarius obsoletus *(results from twenty-four experiments)*

Experimental condition	Per cent mortality	Mean per cent metamorphosed
Substrate-conditioned sea water	1.1	48.5 ± 4.9
Untreated sea water control	1.2	11.2 ± 2.5

b. *Filterability of the substrate factor through membrane filters*

The metamorphosis-inducing substrate factor, if soluble, should pass through a molecular filter unaltered. An experiment was performed similar to those just described except that the substrate-conditioned sea water was divided into two equal parts. One half was filtered through a molecular filter[4] with a pore size of 0.8 μ; the other half was retained as a control. Both water samples were then tested for their metamorphosis-inducing property against a sample of untreated sea water. The resulting metamorphosis of larvae in the unfiltered, substrate-conditioned sea water and the filtered, substrate-conditioned sea water is shown in Table VIII. The mean age of the larvae at the beginning of the experiments was 28.8 days. Twelve and three-tenths per cent of the larvae in the untreated, 46.9% of the larvae in the substrate-conditioned, and 33.0% of the larvae in the filtered, substrate-conditioned sea water metamorphosed within a period of 48 hours. The difference in results between the filtered, substrate-conditioned water and the untreated sea water was significantly different (*P* equal to less than 0.001). The difference between the filtered, substrate-conditioned sea water and unfiltered, substrate-conditioned sea water was not significant since the value of *P* was approximately 0.10.

The experiments showed that the metamorphosis-inducing property of the substratum was at least in part a water-soluble substance; previous evidence has already indicated that it must be relatively stable at temperatures likely to be encountered in nature. One may speculate that the metamorphosis-inducing factor can be adsorbed by the sediment since experiments using the substratum showed

[4] Millipore Filter Corporation, Watertown, Massachusetts.

a consistently higher per cent metamorphosis over those with substrate-conditioned sea water. Such a comparison is not valid, however, without further experiment using similar larvae and sediments. The difference between the mean percentage metamorphosis in the filtered and unfiltered substratum-conditioned sea water, though not statistically significant, may perhaps indicate that under certain circumstances some adsorption may occur either on fine suspended or colloidal particles, which are removed on filtration, or on the filter itself.

c. *Implications of the experiments using substrate-conditioned sea water*

The two series of experiments using substrate-conditioned sea water supply the evidence which makes possible a reasonable hypothesis to explain the nature of the response to the substratum. The experiments suggest that the metamorphosis response of *Nassarius obsoletus* larvae is due to a soluble substance, which is relatively stable, and of biological origin. Such a substance may justifiably be termed a "biologically active" substance. Apparently the larvae perceive this substance without contact with the substratum. The latter conclusion presupposes that the veligers must have chemoreceptors sensitive enough to discriminate between the presence or absence of the metamorphosis-inducing factor in the water.

Table VIII

The effect of filtrating the metamorphosis-inducing property of the substratum through a molecular filter (results from eighteen experiments)

Experimental condition	Per cent mortality	Mean per cent metamorphosed
Substrate-conditioned sea water	1.2	46.9 ± 6.5
Molecular filtered substrate-conditioned sea water (0.8 μ pore diameter)	0.3	33.0 ± 5.6
Untreated sea water	0.3	12.3 ± 3.0

Observations on adult *N. obsoletus* under both laboratory and field conditions indicate a high degree of chemoreception. For example, snails placed in a tank with a small piece of clam meat are able to locate such food very rapidly by testing water through their siphons. Chemical sensitivity has been demonstrated on two European species of the same genus (Henschel, 1932; Weber, 1924). In view of these well-developed chemical sense organs observed in the adults of the genus, it is not too surprising to encounter a well-developed chemoreception in the creeping-swimming stage of *N. obsoletus*.

Two types of chemoreception may be distinguished (Carthy, 1958): a contact chemical sense and an olfactory sense. The distinction is largely that of the concentration required to stimulate. Inasmuch as contact with the substratum by *N. obsoletus* is unnecessary, olfaction, the more sensitive chemical sense, is probably involved in the initial response.

Once the creeping-swimming larva descends to the bottom, further testing of the substratum is possible by contact chemoreceptors. This may provide an explanation for the relatively higher percentage of metamorphosis in those experiments using a substratum over those in which substrate-conditioned water was used.

Biologically active substances encompass a wide variety of materials and have been demonstrated to be of importance in a great diversity of biological activities. The subject has recently been extensively reviewed by Collier (1953) and Lucas (1955). We shall therefore not attempt an extensive summary here. It will suffice to say that the regulation of many marine animal relationships, including commensalism, antibiosis, and predation, have been explained by the demonstration of a biologically active substance.

A large diversity of organic compounds are apparently to be found in the ocean. Collier in 1953 reviewed the types of organic substances which had been found up to that time. Among these he listed N-ethylcarbazol substances ("carbohydrates"), tryosine, tryptophane, rhamnoside, ascorbic acid, and certain other unknown materials, among these a yellow lipoid substance. To these may now be added many other materials which have recently been discovered such as B_{12}-active substances (Burkholder and Burkholder, 1956; Starr *et al.,* 1957), organic acids (Koyama and Thompson, 1959; Slowey *et al.,* 1959), and various algal (Proctor, 1957) and bacterial (Sieburth, 1959) antibiotics.

Clearly, biologically active substances must encompass a wide variety of materials. Collier (1953) believes that organic compounds may originate (1) as degradation products of dead or dying animals and plants, (2) as excretory products, (3) as diffusible metabolites, and (4) from microbial activity. In the case of the metamorphosis response of *Nassarius obsoletus* to bottom sediment we do not yet know the chemical identity nor the exact source of the biologically active material. "A mud or soil with an abundant and varied microflora should contain very nearly the gamut of microbial metabolites . . ." (Hutner and Provasoli, 1951). Moreover, it is not apparent whether more than one substance may be involved in the response of the larvae. The evidence shows no discrete threshold at which metamorphosis occurs, inasmuch as the response of a group of experimental larvae to the metamorphosis-inducing factor is not "all or none." Hence not all creeping-swimming larvae metamorphose after 24 hours, despite the presence of a suitable substratum. Obviously the metamorphosis response to the substratum is not a simple one, nor should simplicity in behavior be expected from larvae, inasmuch as they are in most anatomical and physiological respects fully as complex as adults.

Function and Ecological Significance of the Substratum in Inducing Metamorphosis in Nassarius obsoletus and Nassarius vibex

a. *Adaptive value of the response of larvae to the substratum*

Nassarius obsoletus, which ingests large quantities of bottom material by means of its proboscis, is a deposit feeder. The evidence for this comes from the examination of fecal material and digestive tract contents. Since a crystalline style is present (Jenner, 1956), it is presumed that large amounts of carbohydrates are digested from the microflora within the sediment. Living bivalves of any size or species which have been tried in the laboratory are consistently refused, including those which are frequently associated with *N. obsoletus* (*e.g.,* recently metamorphosed *Venus mercenaria* and juvenile *Gemma gemma*). Subsistence from dead mollusks or Crustacea is possible; indeed, on intertidal flats one frequently encounters aggregations of several hundred *N. obsoletus* feeding on some dead marine organism. Such dead organisms cannot, however, form a principal item of diet because of the large size of the *N. obsoletus* populations found on the intertidal flats.

Observations on another species of the genus *Nassarius* suggest that there is a correspondence between the metamorphosis response to bottom sediment and the adaptive value of such a response. *Nassarius vibex,* which is sometimes found sympatrically with *N. obsoletus,* is a scavenger. It is found much less abundantly than *N. obsoletus,* as would be expected by its trophic position in the community in which it lives. Experimentally we have shown that *N. vibex* does not metamorphose in response to a bottom sediment. A series of eleven experiments resulted in $67.4 \pm 11.8\%$ metamorphosis when sediment was present on the bottom of the experimental containers, whereas $62.9 \pm 11.5\%$ metamorphosed without the presence of sediment. Hence the complex behavior pattern of *N. obsoletus,* which results in the metamorphosis of larvae on a favorable substratum, has considerable adaptive value; such a response seemingly would be of no marked advantage to *N. vibex.*

b. *Ecological significance of the response of* Nassarius obsoletus *to the substratum*

"The continued presence of an animal in an environment depends upon the developmental stage in which it has the least adaptability and this stage, the weakest link of the chain, will normally be found during the breeding period and larval development." (Thorson, 1957, p. 472.) Hence, less stability should be expected of populations of organisms which have long pelagic larval stages. A comparison of arctic bottom communities, where almost all benthic organisms have direct development, with tropical benthic communities, where 75 to 80% of all bottom forms have pelagic larvae, displays no marked difference in the stability of the two (Thorson, 1957). There must exist a means, aside from chance, by which tropical benthic communities maintain their relative stability. Indeed the stability of any bottom community which depends largely on recruitment of pelagic larvae needs explanation.

One interpretation of the relative stability of large benthic populations may be found in the response of larvae to a metamorphosis-inducing factor in the substratum or, more accurately, to a very complex behavior pattern which consists of (1) recognition of a favorable substratum or environment and (2) a response to this favorable substratum, terminating in metamorphosis. This type of response may be expected in benthic forms which are deposit feeders. Our experiments have shown that under optimum laboratory conditions larvae of *N. obsoletus* may metamorphose as early as 17 days after their emergence from the egg capsule, while in the absence of a favorable substratum the termination of planktonic existence may be delayed for as long as 20 days after the creeping-swimming stage has been attained. Although the favorable substratum is come upon accidentally, the larvae may delay their metamorphosis over a period of sufficient time so as to make such an encounter very highly probable. Estuaries, which form the chief habitat for *N. obsoletus,* often have circulations of the type which will carry swimming larvae over long distances upstream along the bottom (Pritchard, 1951). Larvae as they are carried by bottom currents over a particularly suitable region will metamorphose.

Summary

1. Experimental evidence shows that the presence of a favorable substratum has a marked effect on the metamorphosis of *Nassarius obsoletus* larvae. In the presence of a natural substratum the percentage metamorphosis was very significantly greater than in controls without such natural substratum. Larvae postponed metamorphosis for over two weeks when a desirable substratum was not encountered.

2. Certain substrata were preferred over others; a significant difference in the response of *N. obsoletus* larvae to different sediments was demonstrated experimentally. A correspondence exists between substrata preferred under natural conditions and those favored in experiments in the laboratory.

3. The physical properties of a substratum, such as median grain size and sorting, do not directly influence the metamorphosis of *N. obsoletus* veliger larvae. This was demonstrated both experimentally and by field observations.

4. Certain biological properties are important in making the substratum attractive for the metamorphosis of *N. obsoletus* veligers. This was shown by the differences in settlement obtained experimentally when the sediment was treated in various ways. When a sediment was rendered completely abiotic by incineration, no significant difference existed between the percentage metamorphosis on such a substratum and in controls without substratum. If the biological characteristics of the sediment were drastically altered by heating in sea water, the attractiveness of the substratum to metamorphosis by veliger larvae was significantly reduced, but it remained nevertheless significantly more attractive for metamorphosis than a substratum which had been made completely abiotic.

5. The metamorphosis-inducing properties of a substratum may be transferred to the adjacent water, as was demonstrated experimentally.

6. Experimental evidence showed that the metamorphosis-inducing factor from the substratum is probably a water-soluble substance.

7. Inasmuch as the response to a metamorphosis-inducing factor is possible without contact with the substratum, a rather sensitive chemoreception by *N. obsoletus* larvae must be involved. Stimulation may be followed by a complex behavior pattern which includes further testing of the substratum and subsequent metamorphosis.

8 Since *N. obsoletus* is primarily a deposit feeder, the selection of a favorable substratum has considerable adaptive value.

9. The ability to delay metamorphosis and the response to a favorable substratum greatly increase the probability that the larvae of *N. obsoletus* will terminate their pelagic existence in a favorable habitat.

LITERATURE CITED

Ayers, J. C., 1959. The hydrography of Barnstable Harbor, Massachusetts. *Limnol. Oceanogr.*, 4: 448–462.

Burkholder, P. R., and L. M. Burkholder, 1956. Vitamin B_{12} in suspended solids and marsh muds collected along the coast of Georgia. *Limnol. Oceanogr.*, 1: 202–208.

Carthy, J. D., 1958. An Introduction to the Behavior of Invertebrates. George Allen and Unwin, Ltd., London. xiv + 380 pp.

Collier, A., 1953. On the significance of organic compounds in sea water. *Proc. Eighteenth N. A. Wildlife Conf.*, pp. 463–468.

Day, J. H., and D. P. Wilson, 1934. On the relation of the substratum to the metamorphosis of *Scolecolepis fuliginosa* (Claparède). *J. Mar. Biol. Assoc.*, **19**: 655–662.

Harrington, C. R., 1921. A note on the physiology of the ship-worm (*Teredo norvegica*). *Biochem. J.*, **15**: 736–741.

Henschel, J., 1932. Untersuchungen über den chemischen Sinn von *Nassarius reticulata. Wiss. Meeresunters., Abtl. Kiel,* **21**: 133–158.

Hulburt, E. M., 1956. The phytoplankton of Great Pond, Massachusetts. *Biol. Bull.,* **110**: 157–168.

Hutner, S. H., and L. Provasoli, 1951. The phytoflagellates. pp. 27–128. *In:* Biochemistry and Physiology of Protozoa, Vol. I, ed. by A. Lwoff. Academic Press, New York.

Jägersten, G., 1940. Die Abhängigkeit der Metamorphose vom Substrat des Biotops bei Protodrilus. *Arkiv f. Zool.,* **32A** (No. 17): 1–12.

Jenner, C. E., 1956. The occurrence of a crystalline style in the marine snail, *Nassarius obsoletus. Biol. Bull.,* **111**: 304.

Koyama, T., and T. G. Thompson, 1959. Organic acids in sea water. *Preprints Internat. Oceanogr. Congr.* 1959 [New York], AAAS, Washington, D. C., p. 925.

Lucas, C. E., 1955. External metabolites in the sea. Pap. Mar. Biol. and Oceanogr., *Deep-Sea Res.,* Suppl. to Vol. 3: 139–148.

Mortensen, Th., 1921. Studies of the Development and Larval Forms of Echinoderms. Gad, København (Copenhagen). 266 pp.

Mortensen, Th., 1938. Contributions to the study of the development and larval forms of Echinoderms. IV Kgl. *Danske Vidensk. Selsk. Skrifter, Naturv. og Math. Afd. Raekke* 9, **7**: 1–59.

Nyholm, K., 1950. Contributions to the life-history of the Ampharetid, *Melinna cristata. Zool. Bid. Uppsala,* **29**: 79–91.

Pritchard, D. W., 1951. The physical hydrography of estuaries and some applications to biological problems. *Proc. Sixteenth N. A. Wildlife Conf.,* pp. 368–376.

Proctor, V. W., 1957. Studies of algal antibiosis using *Hematococcus* and *Chlamydomonas. Limnol. Oceanogr.,* **2**: 125–139.

Scheltema, R. S., 1956. The effect of substrate on the length of planktonic existence in *Nassarius obsoletus. Biol. Bull.,* **111**: 312.

Sieburth, J. M., 1959. Antibacterial activity of Antarctic marine phytoplankton. *Limnol. Oceanogr.,* **4**: 419–424.

Silén, L., 1954. Developmental biology of Phoronidae of the Gullmar Fiord area (west coast of Sweden). *Acta Zoologica,* **35**: 215–257.

Slowey, J. F., L. M. Jeffrey and D. W. Hood, 1959. Characterization of the ethyl acetate extractable organic material of sea water. *Preprints Internat. Oceanogr. Congr.* 1959 [New York], AAAS, Washington, D. C., p. 935.

Smidt, E. L. B., 1951. Animal production in the Danish Waddensea. *Medd. Komm. Danmarks Fisk. Havunders., Ser. Fiskeri,* **11**(6): 1–151.

Starr, T. J., M. E. Jones and D. Martinez, 1957. The production of vitamin B_{12}-active substances by marine bacteria. *Limnol. Oceanogr.,* **2**: 114–119.

Thorson, G., 1957. Bottom communities (sublittoral or shallow shelf). pp. 461–534. *In:* Treatise on marine ecology and paleoecology, Vol. I: Ecology, ed. J. W. Hedgpeth. *Geol. Soc. Amer., Mem.* **67**. viii + 1296 pp.

Weber, H., 1924. Ein Umdreh und ein Fluchtreflex bei *Nassa mutabilis. Zool. Anz.,* **60**: 261–269.

Wilson, D. P., 1932. On the mitraria larvae of *Owenia fusiformis* Delle Chiaje. *Phil. Trans. Roy. Soc. London, Ser. B,* **221**: 231–334.

Wilson, D. P., 1937. The influence of the substratum on the metamorphosis of Notomastus larvae. *J. Mar. Biol. Assoc.,* **22**: 227–243.

Wilson, D. P., 1948. The relation of the substratum to the metamorphosis of Ophelia larvae. *J. Mar. Biol. Assoc.,* **27**: 723–760.

Wilson, D. P., 1951. Larval metamorphosis and the substratum. *Ann. Biol. (Paris),* **27**: 259–269.

WILSON, D. P., 1952. The influence of the nature of the substratum on the metamorphosis of the larvae of marine animals, especially the larvae of *Ophelia bicornis* Savigny. *Ann. Inst. Oceanogr. (Monaco)*, **27**: 49–156.

WILSON, D. P., 1953a. The settlement of *Ophelia bicornis* Savigny larvae. The 1951 experiments. *J. Mar. Biol. Assoc.*, **31**: 413–438.

WILSON, D. P., 1953b. The settlement of *Ophelia bicornis* Savigny larvae. The 1952 experiments. *J. Mar. Biol. Assoc.*, **32**: 209–233.

WILSON, D. P., 1954. The attractive factor in the settlement of *Ophelia bicornis* Savigny. *J. Mar. Biol. Assoc.*, **33**: 361–380.

WILSON, D. P., 1955. The role of micro-organisms in the settlement of *Ophelia bicornis* Savigny. *J. Mar. Biol. Assoc.*, **34**: 513–543.

ZOBELL, C. E., 1946. Marine Microbiology. Chronica Botanica Co., Waltham, Massachusetts. 240 pp.